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14. ABSTRACT

We have been able to successfully complete the establishment of a 14 institution study group with the enrollment of 550 patients and associated database. Based on known recurrence rates by our statistical support group predicted that there was enough redundancy in the study to support an appropriate power analysis. As such, the MIMS study the five-year follow up completion with data lock anticipated within the next two months. At the moment, there are 69 outstanding Data Clarification Requests (DCRs) and only 7 patients lost to follow up. To date, our interim analysis has been promising and productive. Overall 18 related publications in peer review journals and two patent applications have resulted directly and/or indirectly from the MIMS project. We anticipate being able to address our primary endpoints, and the majority of the secondary endpoints by the conclusion of the study. Additionally, it is notable that the MIMS study per se has led to the successful establishment of several productive and not independently funded research initiatives which are likely to have a "ripple" effect beyond that of the MIMS trial per se.

15. SUBJECT TERMS

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Minimally Invasive Molecular Staging (MIMS) RT-PCR Breast Cancer Study

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OVERALL SUMMARY

A multi-site prospective cohort study was proposed to determine whether a screening procedure to detect occult breast cancer micrometastases using reverse transcriptase-polymerase chain reaction (RT-PCR) in the axillary lymph node (ALN) of patients with primary breast cancer would be a clinically relevant predictor of disease recurrence. In addition to the standard histopathological determination of ALN involvement, an analysis of the multi-marker RT-PCR panel were to be obtained from each of 1,130 breast cancer patients. Of the 1,130 patients, approximately 670 were anticipated to be histopathology negative. These patients are to be followed for five to ten years from initial staging to establish the predictive value of this screening methodology. Furthermore, RT-PCR from sentinel lymph node (SLN), axillary lymph nodes (ALN), whole blood, and bone marrow aspirate samples will be evaluated for their predictive values of disease recurrence.

1. OBJECTIVES

1.1 Primary

The primary objective of the study was to show that the status of occult breast cancer micrometastases in ALN determined by RT-PCR in patients with histologically negative ALN is a clinically relevant predictor of disease recurrence risk. Specifically, based on ALN histology, it was predicted that approximately 30 to 50% of node negative patients in Stages I, IIa, and IIb of breast cancer will be upstaged to the next higher stage level by having an RT-PCR node positive result*, and thus, these patients would experience a 5- to -10 year relapse rate (proportion of patients having relapse within 5- to -10 years from diagnosis) similar to those in the next higher stage level.

1.2 Secondary

1) Evaluate the disease-free survival of the cohort and its association with the RT-PCR results.
2) Determine the sensitivity and specificity of SLN histopathology and RT-PCR results relative to ALN histopathology and RT-PCR results, respectively.
3) Determine the sensitivity and specificity of bone marrow aspirate cytopathology and RT-PCR results relative to ALN histopathology and RT-PCR results, respectively.
4) Assess whether the anticipated superior clinically relevant predictive value of RT-PCR relative to pathology on disease recurrence risk found in ALN (the primary objective) will also be observed in the SLN, whole blood, and bone marrow aspirate samples.

* If at least any one of the three active markers of the multi-panel RT-PCR exhibits node positive result, the patient will be considered to be RT-PCR node positive.

5) Determine the sensitivity and specificity of RT-PCR from whole blood cells relative to ALN histopathology and RT-PCR results.
6) Determine whether RT-PCR is an independent predictor of disease recurrence risk.
7) Evaluate the association between the number of positive markers in the RT-PCR panel and disease severity (as defined by staging).
8) Determine the degree of false negative results by RT-PCR in pathology positive patients in ALN, SLN and bone marrow aspirate samples.
9) Determine the number and specific markers associated with disease recurrence risk.
10) Identify baseline risk factors that may affect the predictive value of RT-PCR on disease recurrence.
11) Assess the types and incidence rate of adverse events associated with sentinel node dissection and bone marrow aspiration.

2. BACKGROUND AND RATIONALE (as discussed in the original proposal)

2.1 Background of Current Methodology for Detection of Nodal Involvement

2.1.1 ALN Involvement in Breast Cancer Prognosis

Breast cancer is a leading cause of cancer death among American women [1]. Lymph node status remains the most valuable prognostic indicator for breast cancer, with an inverse relationship existing between the number of positive lymph nodes for cancer and prognosis [1-3]. The current method of staging for breast cancer is to perform a formal ALN dissection in these patients with an estimated annual cost of $1.5 billion [4-8]. Despite this aggressive surgical approach to determine lymph node involvement, the staging of breast cancer is clearly inaccurate using current methods of histopathologic analysis of lymph nodes. Given that only a small portion of the node is examined, there is uncertainty as to whether the specific sample evaluated is appropriate. Up to 25% of histologically negative nodes contain occult micrometastases [9]. Furthermore, as many as 30% of patients with pathologically negative lymph nodes ultimately develop recurrent disease [10].

2.1.2 Current Methodology

The current practice for staging primary breast cancer patients involves histologic examination of paraffin-blocked hematoxylin and eosin (H&E) stained lymph nodes from breast cancer patients obtained via formal ALN dissection of sentinel lymph node biopsy. The lymph nodes are sectioned in half and usually the pathologist examines one or two sections from each node for metastases. Thus, if there is not a metastatic cell in that micron section of the lymph node, the entire lymph node is presumed to not contain metastatic disease. A number of studies have evaluated, and attempted to increase, the accuracy of histology by performing multiple sections and adding immunohistochemical staining [9; 11-

14]. Although this type of meticulous sectioning and staining is too cumbersome and costly to be performed on a regular basis. For ALND, it is often performed on a sentinel lymph node if the H+E is negative. The development, therefore, of a more sensitive and cost effective approach to examining lymph nodes for metastases becomes relevant.

2.2 Background of Minimally Invasive Molecular Staging (MIMS) Methodology

2.2.1 Multi-marker Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Recent identification of genes expressed in breast cancer combined with advances in molecular oncology have provided the opportunity to establish more sensitive, specific, and potentially cost effective ways of identifying metastatic disease. RT-PCR as a screening modality combines the powerful capability of PCR to amplify and detect small amounts of DNA from a specific gene with the ability to use some or all of the tissue being evaluated.

Thus, RT-PCR should have a marked increase in sensitivity as compared to the relatively small amount of tissue which is actually sampled when one or two sections of each node are used in H&E staining. In support of this premise, previous work by several investigators has shown RT-PCR to be capable of detecting one cancer cell expressing a unique gene in $1x10^6$ normal lymph node cells [15,16].

Currently, no single marker is uniformly expressed in all breast cancers. A RT-PCR panel based on multiple markers, therefore, may provide the most effective methodology for identifying micrometastases. A similar multi-marker RT-PCR method has been used to detect circulating melanoma cells, and was shown to be more reliable and more sensitive than a single-marker assay [17]. A number of gene markers, including keratin-19 [18-24], c-myc [25-27], and prolactin inducible protein (PIP) [28-32], are preferentially expressed in breast cancer compared to normal tissues. Preliminary data from our study of a multi-gene panel based on these genes show that this panel provides a more sensitive and possibly more cost effective assay for the presence of micrometastatic disease [33-35].

2.2.2 SLN Biopsy and Bone Marrow Aspirate

The current standard method of obtaining tissue for staging breast cancer is to perform a formal axillary dissection. In addition to its inaccuracies, the need for axillary dissection is controversial. With the increased use of screening mammography, both the size of the tumors as well as the risk of developing metastases in ALN are decreasing. Additionally, studies have established that there is no improvement in survival in patients who undergo axillary dissection, and it may only serve to decrease the rate of recurrence in the axilla [6-7]. This, coupled with potential short and long term complications including fluid

collections, bleeding, arm swelling, chronic pain, and lymphatic malignancy, in addition to an overnight stay in the hospital [36], is convincing data to support less invasive techniques for breast cancer staging. Because of its minimal tissue requirements, RT-PCR screening could be effectively combined with new and promising minimally invasive staging techniques for breast cancer such as (1) SLN biopsy [37-42] or (2) bone marrow aspirate [43-46].

The SLN biopsy, introduced by Morton et al [40], was originally based upon the theory that melanomas drain to discreet identifiable lymphatic basins and that the first nodes in these basins, the sentinel node(s), will harbor the metastatic disease if it is present [41, 42]. A study on SLN dissection for breast, first described by Giuliano in 1994 [37, 38], showed that the SLN biopsy could accurately identify the ALN draining the primary breast tumor, which is the node most likely to contain tumor cells that have spread to the axilla.

Breast tumor cell detection (TCD) in bone marrow by means of immunocytochemistry was first described in 1981 by Dearnaley, *et al* [45] and is considered an indicator of subsequent metastasis not only to the lymph nodes but also to distant sites [43, 46]. More recently, in retrospective series involving 792 patients [44], TCD was found to be a prognostic indicator independent of nodal status. These findings therefore raise the question as to whether this simple and inexpensive procedure of bone marrow aspiration could replace axillary dissection in some subgroups of patients with breast cancer whose ALN are expected to be negative.

2.3 Rationale

Establishing the clinical relevance of staging based on RT-PCR positive lymph nodes is the next essential step in order to validate this screening methodology. To date, no prospective study exists which evaluated the association between occult metastases and worsened survival. It is unknown at this time if patients with RT-PCR positive lymph node status will have recurrence and survival rates similar to their original stage. Previous retrospective studies suggest that the biologic behavior of patients with occult micrometastases (discovered by step sectioning and immunohistochemical staining) is similar to those with histologically positive nodes, and would suggest that RT-PCR positive patients may also have a worse prognosis [9; 11-14]. Further long-term follow-up of patients with RT-PCR positive and histologically negative specimens will clearly be required to determine the clinical relevance of RT-PCR staging.

If the results of this trial support our hypothesis, the impact would be twofold: establishing a more sensitive methodology for detecting clinically relevant micrometastatic disease in breast cancer patients and validating less invasive tissue sampling techniques which could be used with RT-PCR screening. Individually these outcomes have clinical significance, combined they could represent a major advance in the management of breast cancer. Staging of breast

cancer could potentially change from involving very invasive surgery to minimal procedures with a gain in accuracy and sensitivity. Ultimately, it would translate into improving survival by identifying those patients that we have heretofore been unable to identify as high-risk patients and treat them aggressively, or more appropriately observe early stage disease. With an estimated 175,000 new invasive breast cancer cases diagnosed in 1999 alone [47], the results of this proposal could have an enormous impact on the surgical management of breast cancer.

3. ORIGINAL STUDY PLAN

A prospective cohort study design was adopted where, upon recruitment, eligible participants with Stage I, IIa or IIb breast cancer were requested to consent to tissue sampling from ALN, SLN, bone marrow aspirate and whole blood. Tissue sampling was accomplished during the planned surgical resection while under anesthesia. Pathology was conducted on lymph nodes at each participating clinical site and RT-PCR analyses was conducted on all tissue submitted to the central RT-PCR laboratory. Staging was done according to the standard guidelines established by the American Joint Committee on Cancer (AJCC), including histopathology of ALN, but not RT-PCR. All RT-PCR analysis was conducted at the Medical University of South Carolina (MUSC) Central RT-PCR Laboratory blinded to the pathology results. Treatment decisions followed the current standard of care without the knowledge of, or reference to, the RT-PCR analysis and was made by the treating physician at each clinical site.

3.1 Study Subjects

An approximate sample size of 670 participants who are pathology node negative in the ALN was initially required to evaluate the primary hypothesis. If it is assumed that the 670 pathology node negative patients represent about 60% of all Stages I-II breast cancer cases, approximately 1,130 participants are required for the study. The RT-PCR results of the 460 ALN positive (by histopathology) participants would enable testing of some of the secondary hypotheses with adequate power. Based on the initial study, it was anticipated that at least 95% of eligible patients will consent to RT-PCR analysis, at a minimum, on their ALN sample.

To reduce the chance of spurious results, participants who had previously received treatment for breast cancer or participants who had been successfully treated for other malignancies within 10 years were excluded.

3.2 Number of Study Sites

Fourteen clinical centers with expertise in the multi-disciplinary management of patients diagnosed with breast cancer participated in the study. Each clinical center had a Principal Investigator, and/or Surgeon, Pathologist and Clinical

Research Coordinator.

5.0 CURRENT CLINICAL TRIAL STATUS

Patient enrollment ended December 2001 with a total of 550 patients entered onto study. This number was significantly less that the original anticipated patient enrollment despite a vigorous recruitment effort involving all 14 institutions involved. Statistical analysis performed based on known recurrence rates by the TCIG group predicted that there was enough redundancy in the study to support an appropriate power analysis. As such, the MIMS study enrollment was concluded in December 2001. A current clinical site status summary is noted as follows:

5.1 Clinical Site Status:

Clinical Site	Status	# enrolled	# complete	# in follow up	# lost to follow up	# local rec.	# distant/ metastatic rec.	# w/ 2nd cancer	# died
MUSC (001)	Closed 01/2007	130	130	0	2	0	4	7	3
Howard University, Washington, DC (002)	Enrollment Completed 12/31/2001; Follow-up	12		9	0	0	0	0	0
Florida Health Science Center, Jacksonville (024)	Closed 06/2004	6	6	0	0	0	0	1	1
University of Alabama at Birmingham (025)	Enrollment Completed 12/31/2001; Follow-up	111	103	8	0	2	1	1	1
Spartanburg Regional Healthcare System (027)	Closed 06/2004	29	29	0	0	1	0	2	0
Washington University, St. Louis, MO (028)	Enrollment Completed 12/31/2001; Follow-up	59	54	5	4	0	1	0	0
Medical College of Georgia (029)	Closed 12/2004	4	4	0	1	0	0	0	0
University of Tennessee (047)	Closed 06/2004	25	25	0	0	1	2	3	1
Palmetto Health Alliance (058)	Enrollment Completed 12/31/2001; Follow-up	3	0	3	0	0	0	0	0
Medical College of Wisconsin (061)	Closed 01/2007	7	7	0	0	0	0	0	0
Royal Melbourne Hospital, Australia (111)	Enrollment Completed 12/31/2001; Follow-up	74	71	3	0	1	1	2	2
Lincoln and Louth County Hospital, England (113)	Enrollment Completed 12/31/2001; Follow-up	31	14	17	0	1	1	1	1
St. Vincent's, Australia (116)	Enrollment Completed 12/31/2001; Follow-up	16	13	3	0	1	0	0	1
St. Vincent's Hospital, Ireland (117)	Enrollment Completed 12/31/2001; Follow-up	43	23	20	0	0	0	0	0
Total:		**550**	**482**	**68**	7	7	**10**	**17**	**10**

Study Status and Follow-ups:

There are currently 68 subjects in follow-up. There have been 10 deaths and 7 subjects reported as "lost to follow-up".

In the last three months, we have received approximately 159 follow-up forms. Subject follow-up forms indicating local or distant disease recurrence or new malignancy are monitored using a copy of the pathology report. Data management generates data queries as a result of missing data points or incorrect data points entered on the follow-up CRFs. At this time there are approximately 69 outstanding Data Clarification Requests (DCRs).

We have continued to track outstanding follow-up forms and have sent repeated reminders to the sites asking for delinquent data and query responses. We will continue to make these requests through the end of January 2007 at which time we will then proceed with database locking. **A final outcomes analysis is currently pending, awaiting the database locking finalization.**

6. SUMMARY OF PROGRESS TO DATE

6.1 PROGRESS RELATED DIRECTLY TO THE MIMS TRIAL. Our original observations leading the initiation of the MIMS study were that RT-PCR based screening methodology using a multimarker based analysis had an increased sensitivity and could be potentially cost effective as an analytic (***1.*** *Lockett et al, Detection of occult breast cancer micrometastases in axillary lymph nodes using a multi-marker RT-PCR panel. Journal of the American College of Surgeons, 187: 9-16, 1998.* ***2.*** *Lockett et al, Efficacy of RT-PCR Screening for Micrometastatic Disease in Axillary Lymph Nodes of Breast Cancer Patients. American Surgeon, 64: 539- 544, 1998*). With the successful DOD funding of the MIMS study, we initially turned our attention to addressing the issue of developing a more robust and quantitative marker set to be applied with the MIMS trial. This body of work occurred in parallel to the site identification and initiation of the trial per se. We were able to identify with collaborators in British Columbia two novel marker (Prolactin-inducible protein and Prostate Specific Ets factor) and validate a six marker set which utilized real-time PCR technology (***3.*** *Clark JW et al, A role for prolactin-inducible protein (PIP) as a marker of human breast cancer metastasis. British Journal of Cancer, 81:1002-8, 1999.* ***4.*** *Mitas et al, Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multi-gene marker panel. International Journal of Cancer, 93:162-171, 2001,* ***5.*** *Mitas et al, Prostate-Specific Ets (PSE) factor as a novel marker for detection of breast cancer metastatic disease. British Journal of Cancer, 86:899-904, 2002*). Additionally, based on the original novel concepts which we had been able to concieve, validate and publish prior to DOD funding, we were able to successfully patent the multimarker concept for application to breast cancer diagnostics (*US Patent #6,037,129. Multi-marker RT-PCR panel for detecting metastatic breast cancer. Issued March 14, 2000.* ***Cole DJ,*** *Baron PL, O'Brien PH. Licensed 11/04*). This patent was licensed in November 2004 by Cephiad Industries for the development of a breast cancer molecular analytic. A second patent was ultimately filed based on data resultant from the MIMS study marker analysis (*Mitas M, Mikhitarian K.* ***ColeDJ****. U.S. Utility Patent Application. Use of the XAG gene for detection of metastatic breast and pancreatic cancer in lymph nodes. May 2005*). Although we are currently awaiting the final database lock in order to perform the appropriate analysis, we have been able to

make several cogent observations based in interim data. Utilizing traditional prognostic indicators documented in our MIMS database, we were able to define a statistically significant relationship between molecular positive nodal disease and predicted outcome. Although not definitive, these findings are consistent with our primary hypothesis were presented at the Southern Surgical Association meeting December 2003, and subsequently published in our leading surgical journal (**6.** *Gillanders WE et al, Detection of micrometastatic disease in axillary lymph nodes in pathology-negative breast cancer patients correlates with traditional prognostic indicators: An interim analysis of a prospective multi-institute breast cancer cohort study. Annals of Surgery, 239:828-840, 2004.*). We have subsequently performed a significant amount of work looking at the sentinel lymph node and bone marrow specimen subsets. The SLN data analysis was supportive of the hypothesis that sentinel lymph node biopsy concept was valid at the molecular level of sensitivity (**7.** *Mikhitarian et al, Molecular analysis improves the sensitivity of breast sentinel lymph node biopsy: results of a multi-institutional prospective cohort study. Surgery. 138(3):474-81, 2005*). The one disappointing data set to date is the bone marrow analysis which has not been informative despite careful IHC and molecular analysis. Although there might be useful bone marrow related data once the final analysis is performed, it would appear that the rudimentary method of isolation a marker analysis utilized for this will preclude any significant diagnostic insights. Finally, with the validation of the 6 marker breast detection set, we were able to establish a new collaboration with the University of Pittsburgh with the intention of combining our marker expertise with their multiplex PCR expertise in order to develop a rapid marker analytic which could potentially be applied real time to allow definitive analysis of SLN status at the time of surgery. To date this has resulted in a seminal publication in Annals of Surgery (**8.** *Hughes et al, A Rapid, Fully-Automated, Molecular-Based Assay Definitively Analyzes Sentinel Lymph Nodes for the Presence of Metastatic Breast Cancer. Annals of Surgery 243:389-398, 2006*) with beta testing ongoing via an NIH SBIR funding mechanism (*NIH SBIR CA-099123 Phase II (Cephiad Industries, Co-PI-Hughes, **Cole DJ**), QT-PCR detection of SLN micrometastases. 7/2004-6/2006, $152,110 (annual total costs*).

6.2 PROJECTS RESULTING FROM THE PRIMARY MIMS STUDY: LUNG CANCER ANALYTICS. With the development of a significant molecular diagnostic expertise, we worked collaboratively with clinical colleagues in related fields which have led to several successful funded projects. As these clearly would not have occurred without the presence of the original MIMS project, it is appropriate to note these related projects in this progress report. With an initial identification of a lung cancer relevant marker set (***9.*** *Mitas et al, Real-time reverse transcription-PCR detects KS1/4 mRNA in mediastinal lymph nodes from patients with non-small cell lung cancer. Clinical Chemistry, 49:312-315, 2003.)* we were able to establish a pilot study evaluating the clinical relevance of molecular positive lymph nodes in the mediastinum of lung cancer patients. This is relevant as this discernment is critical to surgical decision making. This was subsequently funded via an NIH R21/33 mechanism and the five year study will be completed this coming year (NIH 1R21CA097875-01 (*Wallace PI, Co-PI- Mitas M, Gillanders WE, **Cole DJ**), Detection of occult mediastinal lymph node metastases. 7/2002-6/2007, $152,110 (annual total costs*). Additionally, given the novelty of the

markers involved were were able to file for a patent (*Mitas M, **ColeDJ**, Gillanders WE. U.S. Utility Patent Application. Use of the KS1/4 gene for detection and/or diagnosis. December 2004.*) The preliminary data from these studies is quiet promising and has recently been published (***10.** Wallace et al. Accurate Molecular Detection of Non-Small Cell Lung Cancer Metastases in Mediastinal Lymph Nodes Sampled by Endoscopic Ultrasound-Guided Needle Aspiration. Chest, 127:430-437, 2005.*) in a high impact journal in the field.

6.3 PROJECTS RESULTING FROM THE PRIMARY MIMS STUDY: PERIPHERAL BLOOD TUMOR CELL DETECTION. The initial MIMS trial included PBL analysis which has resulted in a manuscript currently submitted (***11.** Mikhitarian K et al, Detection of mammaglobin mRNA in peripheral blood is associated with high grade breast cancer: interim results of a prospective cohort study. (Submitted, Clinical Cancer Research 2006*) suggesting the utility of mammaglobin for this marker set analysis. It was clear early on in the MIMS study, however, that a more sophisticated isolation technology would be needed in order to push this particular field forward. So, in parallel to the ongoing MIMS analysis, we were able to establish techniques for the efficient isolation and detection of circulating tumor cells (CTC) from the peripheral blood of metastatic breast cancer patients. Although not novel conceptually, we were the first group to define the detection of CTC using molecular technology as opposed to immunohistochemical staining (***12.** Baker MK et al, Molecular detection of breast cancer cells in the peripheral blood of advanced stage breast cancer patients using multi-marker real-time RT-PCR and a novel porous barrier density gradient centrifugation technology. Clinical Cancer Research, 9: 4865-71, 2003.*) and were able to perform a pilot study with the detection of CTC in 70% of these patients. We had similar success with lung cancer patients (***13.** Mitas M et al, Lunx is a superior molecular marker for detection of non-small cell lung cancer in peripheral blood. Journal of Molecular Diagnostics. 5:237-42, 2003.* With these pilot studies we were able to obtain NIH K23 funding for this project, and this has subsequently resulted in a successful R01 funded grant (NIH-1K23CA934191A1 *(Gillanders WE, **Award Mentor- Cole DJ**) Molecular Detection of Breast Cancer in Peripheral Blood. 7/02-6/05. $176,110 (annual total costs.).*

6.4 SUMMARY. We have been able to successfully complete the establishment of a 14 institution study group with the enrollment of 550 patients and associated database. Although this number was significantly less that the original anticipated patient enrollment despite a vigorous recruitment effort involving all 14 institutions involved, statistical analysis performed (based on known recurrence rates by our statistical support group) predicted that there was enough redundancy in the study to support an appropriate power analysis. As such, the MIMS study enrollment was concluded in December 2001. We are nearing the five-year follow up completion with data lock anticipated within the next two months. At the moment, there are 69 outstanding Data Clarification Requests (DCRs) and only 7 patients lost to follow up. The currently delay in data base lock is do to these outstanding DCR. To date, our interim analysis has been promising and productive. We anticipate being able to address our primary endpoints, and the majority of the secondary endpoints by the conclusion of the study. Additionally, it is notable that the MIMS study per se has led to the successful establishment

of several productive and not independently funded research initiatives which are likely to have a "ripple" effect beyond that of the MIMS trial per se.

7. RESULTANT PUBLICATIONS TO DATE (in sequential order of publication)

1. Lockett MA, Metcalf JS, Baron PL, O'Brien PH, Elliot BM, Robison JG, **Cole DJ.** Efficacy of RT-PCR Screening for Micrometastatic Disease in Axillary Lymph Nodes of Breast Cancer Patients. American Surgeon, 64: 539- 544, 1998.
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prognostic indicators: An interim analysis of a prospective multi-institute breast cancer cohort study. Annals of Surgery, 239:828-840, 2004.

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8. RESULTANT PATENTS/APPLICATIONS

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